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(54) Title: ANTIGEN DELIVERY SYSTEM

(57) Abstract

Provided is a method of stimulating a class I–restricted immune response to a protein of interest or antigenic portion thereof in a host, as well as a protein delivery vehicle for use in the method. A nucleic acid molecule encoding the protein of interest or antigenic portion thereof is introduced into an avirulent *Salmonella* spp., such that the resulting *Salmonella* encodes a chimeric protein comprising the protein of interest or antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof. This resulting *Salmonella* can be introduced into a host, in which the *Salmonella* will inject the chimeric protein into the cytosol of the cells of the host. The injection of the chimeric protein results in the stimulation of a class I–restricted immune response to the protein of interest or antigenic portion thereof in the host.

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ANTIGEN DELIVERY SYSTEM

This application claims priority of U.S. Provisional Patent Application No. 60/047,955, filed May 29, 1997, and is a continuation-in-part of U.S. Patent Application Serial No. 08/987,691, filed December 9, 1997.

The subject invention was made with support under Public Health Service Grant Nos. AI30492 and GM52543 of the National Institutes of Health.

FIELD OF THE INVENTION

The subject invention is directed generally to immune responses, and more particularly to a method of stimulating a class I-restricted immune response in a host and to a protein delivery system for use in such a method.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for each of these publications are provided at the end of the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

Most infectious disease agents gain entrance to the host through a mucosal surface, therefore the first line of defense is the mucosal immune system. In fact, protection against many microorganisms better correlates with local than systemic immune responses (Galan et al. 1986; Galan and Timaney 1985). The use of non-replicating antigens to stimulate mucosal immune responses has been hampered by the lack of adjuvants that effectively induce secretory immunity. Live, replicating antigens are known to better stimulate mucosal immunity partly because they tend to persist longer (Ganguly and Waldman 1980). Avirulent strains of *Salmonella typhimurium* endowed with the ability to express cloned genes from other pathogens have been used to stimulate a generalized mucosal immune response against the

recombinant virulence antigens (Doggett and Curtiss 1992; Curtiss et al. 1988; Curtiss et al. 1990; Galan et al. 1988). This approach is based on the fact that *S. typhimurium* invades and proliferates in the gut-associated lymphoid tissue (GALT) (Carter and Collins 1974) and that antigens delivered into the GALT lead to an immune response at other mucosal sites (Cebra et al. 1976).

After oral ingestion, *Salmonella typhimurium* penetrates the cells of the intestinal epithelium (Takeuchi 1967). Once internalized, *Salmonella* are translocated through the epithelial cells to the lamina propria where they are later taken up by macrophages. During the translocation process, *Salmonella* transit inside endocytic vesicles where they undergo limited replication. This is unlike other invasive pathogens, such as *Shigella spp.* or *Listeria monocytogenes*, which escape the endocytic vesicles shortly after internalization and actively replicate in the cell cytosol.

The compartment in the eukaryotic cell in which a bacterium resides is very important when it is being considered as an antigen delivery vehicle, because its location will largely determine whether the antigen will be recognized in association with MHC class I or class II molecules. Antigens presented in the context of class I MHC molecules will predominantly induce cytotoxic T cells (T_{ctl}), while antigens recognized in association with MHC class II molecules will be more likely to stimulate T helper cells (T_H) (Harding et al. 1988; Chain et al. 1988; Allen 1987). This is of great importance in vaccine design since protection against different infectious agents requires different types of immune responses. Thus in general terms, T_{ctl}'s play a key role in protection against most viral and some intracellular bacterial pathogens while T_H's are more important in responses against exogenous antigens that enter the

processing cells (expressing class II molecules) by endocytosis (Long and Jacobson 1989; Long 1989; Kaufman 1988).

It has been established that processing of exogenous antigens involves endocytosis, partial degradation within the endocytic vacuole, and binding to class II MHC molecules. Processing of class I-restricted antigens also appears to involve proteolysis and recognition of antigen-derived peptides bound to MHC class I molecules, although this processing is not secondary to endocytosis. Rather, antigens synthesized within host cells (e.g., viral proteins), or antigens derived from intracellular bacteria that have the ability to exit the endocytic vacuole (e.g., *Listeria monocytogenes* and *Shigella spp.*), are processed and then preferentially associate with MHC class I molecules (Long and Jacobson 1989; Kaufman 1988).

Even though humoral (in particular mucosal) immune responses are an important part of the protective mechanisms against pathogens, it is clear that for efficient protection, cell-mediated immunity is often essential. This is particularly so when the pathogen in question is a virus or an intracellular bacterium. In many of these cases, class I restricted-immune responses are thought to be crucial for protection. This type of immune response is stimulated by proteins that are newly synthesized (e.g., viral antigens) or that otherwise gain access to the cytosol of the infected cell (e.g., *Listeria* antigens). *S. typhimurium* has the ability to invade (enter) mammalian cells. Unlike other facultative intracellular pathogens such as *Listeria* or *Shigella spp.*, which gain access to the cytosol shortly after entry, *Salmonella spp.* remain inside the endocytic vesicle throughout their entire intracellular life cycle. Although there are some exceptions to this generalization (Aggarwal et al. 1990; Flynn et al. 1990), it appears that *Salmonella* is not very efficient at stimulating class I-restricted immune responses, which are known to

be crucial for protection against viruses and a variety of intracellular pathogens (Gao et al. 1992; Yang et al. 1990). This has been clearly demonstrated using avirulent *Salmonella* strains expressing different antigens from influenza virus. In a series of very elegant studies (Brett et al. 1993; Tite et al. 1990a; Tite et al. 1990b), it was shown that mice vaccinated with avirulent strains of *Salmonella* expressing the influenza virus NP failed to mount a significant class I-restricted T cell response against the NP, although they successfully induced class II-restricted responses. On the contrary, class I-restricted responses against the NP were readily demonstrated in mice infected with the virus. As a consequence of this failure, recombinant *Salmonella* vaccine strains failed to protect mice against influenza virus challenge since in this model of NP immunization, protection is largely dependent on nucleoprotein-specific class I-restricted $CD8^+$ cells.

An essential feature of the pathogenesis of *Salmonella spp.* is their ability to stimulate a variety of host-cell responses (reviewed in Galan and Bliska 1996). These responses are largely dependent on the type of cell engaged by the bacteria. For example, in non-phagocytic cells such as those of the intestinal epithelium, *Salmonella spp.* induce profound cytoskeletal rearrangements, membrane ruffling and macropinocytosis which ultimately result in bacteria internalization. In macrophages, on the other hand, *Salmonella spp.* induce programmed cell death (Chen et al. 1996). Essential for the stimulation of these responses is the function of a specialized protein secretion system encoded at centisome 63 of the bacterial chromosome (reviewed in Galan 1996). This protein secretion system, termed type III, directs the export of a number of proteins, some of them with presumed effector function. Characteristic features of this protein secretion system, which has also been identified in several other animal and plant pathogenic

bacteria, include: 1) the absence in the secreted proteins of a typical, cleavable, *sec*-dependent, signal sequence; 2) the requirement of several accessory proteins for the export process; 3) the export of the target proteins through both the inner and outer membranes; and 4) the requirement of activating extracellular signals for its full function (reviewed in Galan 1996). Studies of pathogenic *Yersinia spp.* have established that a similar type III secretion apparatus directs the translocation into the host cells of a number of putative effector proteins such as the bacterial outer proteins YopE, YopH, YopM and YpkA (Rosqvist et al. 1994; Sory and Cornelis 1994; Persson et al. 1995; Sory et al. 1995; Hakansson et al. 1996). Such translocation is thought to occur in a polarized manner in which proteins are transferred directly from the bacteria to the host cells without secretion into the infection medium. A notion has therefore emerged that protein translocation into host cells is perhaps the main function of this type of protein secretion system. This hypothesis is further supported by the observation that type III protein secretion systems have always been identified as essential determinants involved in intimate interactions of bacterial pathogens with their hosts (reviewed in Galan and Bliska 1996).

Several *Salmonella* proteins that are exported through this pathway have been identified although it is not known which, if any, of these proteins is translocated into host cells.

A need exists for new methods for stimulating class I-restricted immune responses.

SUMMARY OF THE INVENTION

The subject invention addresses this need by providing a method for stimulating a class I-restricted immune response to a protein of interest or antigenic portion thereof in a host. The method comprises:

introducing a nucleic acid molecule encoding a protein of interest or antigenic portion thereof into an avirulent *Salmonella* spp., the nucleic acid molecule being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and introducing the resulting *Salmonella* spp. into a host, wherein the resulting *Salmonella* spp. injects the chimeric protein into the cytosol of cells of the host thereby stimulating a class I-restricted immune response to the protein of interest or antigenic portion thereof in the host.

The invention further provides a protein delivery vehicle which comprises: an avirulent *Salmonella* spp. encoding a chimeric protein, the chimeric protein comprising a protein of interest or an antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof.

Further provided is a chimeric protein which comprises: a first amino acid sequence of an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and a second amino acid sequence of a protein of interest or antigenic portion thereof introduced into the first amino acid sequence of the injectable protein or injectable portion thereof.

Also provided is a chimeric nucleic acid molecule which comprises: a first nucleic acid sequence encoding an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and a second nucleic acid sequence encoding a protein of interest or antigenic portion thereof introduced into the first nucleic acid sequence of the injectable protein or injectable portion thereof, the second nucleic acid sequence being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic

portion thereof and the injectable protein or injectable portion thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1 illustrates the insertion site of the influenza virus nucleoprotein epitope at the permissive site of SptP located between the two predicted independent domains of this protein;

Figs. 2 and 3 illustrate antigen presentation by RMA or TAP-deficient RMA-S cells infected with *S. typhimurium*. RMA (Fig. 2) or RMA-S (Fig. 3) cells were infected with wild type, *aroA*, *aroA sptP* or *sipD S. typhimurium* strains bearing a plasmid expressing SptP-$IVNP_{366-374}$ or SptP-$LCMVNP_{118-126}$ prior to exposure to 12.164 hybridoma cells. The concentration of IL-2 secreted into the medium was determined by ELISA. When indicated, control RMA and RMA-S cultures were treated with 1 μM of $IVNP_{366-374}$ peptide, LCMV $NP_{118-126}$ peptide or were infected with influenza virus A/PR/8/34. Error bars depict SEM. Results are representative of three independent experiments;

Fig. 4 illustrates the lysis of $IVNP_{366-374}$ peptide-sensitized EL-4 cells by re-stimulated splenocytes from mice immunized with the *aroA sptP S. typhimurium* strain SB824 expressing SptP-$IVNP_{366-374}$, an irrelevant peptide (SptP-$LCMVNP_{118-126}$), or non-translocatable InvJ-$IVNP_{335-498}$. Control mice were vaccinated intraperitoneally with live influenza virus A/PR/8/34 or recombinant vaccinia virus expressing influenza nucleoprotein (Vaccinia NP)(Smith et al. 1987). Effector to target (E:T) ratios are indicated. Error bars depict SEM. Results are representative of three independent experiments; and

Figs. 5 and 6 illustrate the immune response of mice immunized with avirulent *S. typhimurium* expressing SptP-LCMVNP$_{118-126}$. Fig. 5: Survival of vaccinated mice following LCMV intracerebral challenge inoculation. Groups of BALB/c mice were alternatively vaccinated with the avirulent *aroA sptP S. typhimurium* strain SB824 expressing SptP-LCMVNP$_{118-126}$, or SptP fused to an irrelevant epitope (SptP-IVNP$_{366-374}$), an avirulent *S. typhimurium sipD* strain, defective in translocation but not in secretion of SptP-LCMVNP$_{118-126}$ or mock vaccinated with broth. Fig. 6: Percent specific lysis by in vitro re-stimulated splenocytes from mice inoculated with *aroA sptP S. typhimurium* expressing SptP-LCMVNP$_{118-126}$ or SptP fused to an irrelevant peptide (SptP-IVNP$_{366-374}$). Control mice were vaccinated by intraperitoneal inoculation with live LCMV. Effector to target (E:T) ratios are indicated. Error bars depict SEM. Results are representative of three independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, an "avirulent" *Salmonella* refers to a *Salmonella* which is not capable of causing disease in the host to which it is administered.

As further used herein, a "*Salmonella* encoding" refers to a *Salmonella* which has nucleic acid therein which encodes the referenced protein, either as extrachromosomal nucleic acid or as nucleic acid incorporated into the genome of the *Salmonella*.

The subject invention provides a method for stimulating a class I-restricted immune response to a protein of interest or antigenic portion thereof in a host. The method comprises: introducing a nucleic acid molecule encoding a protein of interest or antigenic portion thereof into an avirulent *Salmonella* spp., the nucleic acid molecule being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic portion thereof and an injectable protein which

is a target of a type III secretion system or an injectable portion thereof; and introducing the resulting *Salmonella* spp. into a host, wherein the resulting *Salmonella* spp. injects the chimeric protein into the cytosol of cells of the host thereby stimulating a class I-restricted immune response to the protein of interest or antigenic portion thereof in the host.

The invention further provides a protein delivery vehicle which comprises: an avirulent *Salmonella* spp. encoding a chimeric protein, the chimeric protein comprising a protein of interest or an antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof. A host into which the protein delivery vehicle has been introduced is also provided. Suitable hosts include those in which a class I-restricted immune response is desirable, including, for example, humans.

Further provided is a chimeric protein which comprises: a first amino acid sequence of an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and a second amino acid sequence of a protein of interest or antigenic portion thereof introduced into the first amino acid sequence of the injectable protein or injectable portion thereof.

Also provided is a chimeric nucleic acid molecule which comprises: a first nucleic acid sequence encoding an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and a second nucleic acid sequence encoding a protein of interest or antigenic portion thereof introduced into the first nucleic acid sequence of the injectable protein or injectable portion thereof, the second nucleic acid sequence being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic portion thereof and the injectable protein or injectable portion thereof.

In each of the above embodiments of the invention, the protein of interest is selected based on the desirability of stimulating a class I-restricted immune response thereto. The many pathogens known to exist and yet to be discovered are examples of sources of "proteins of interest". For example, microbial proteins may be of interest. These include, for example, proteins from bacterial, viral, parasitic, and protozoan pathogens. This list is not inclusive, and the concept of the subject invention is equally applicable to any "protein of interest" as defined above.

Furthermore, the particular injectable protein can be any protein which is a target of a type III secretion system (see Galan 1996; Rosqvist et al. 1994; Sory and Cornelis 1994; Persson et al. 1995; Sory et al. 1995; Hakansson et al. 1996; and Galan and Bliska 1996 for discussions of type III secretion systems suitable for use in the subject invention). Any type III secretion systems known to exist and yet to be discovered are examples of "type III secretion systems" in accordance with the subject invention. For example, currently known targets of bacterial type III secretion systems include the SptP, SipA, SipB, SipC, SipD, InvJ, SpaO, AvrA, and SopE proteins of *Salmonella*, the Yop and Ypk proteins of *Yersinia* (for example, YopE, YopH, YopM and YpkA), the Ipa proteins of *Shigella*, and the ExoS proteins of *Pseudomonas aeruginosa*.

Standard laboratory techniques known in the art of recombinant DNA and bacterial genetics can be used to construct chimeric proteins and to introduce a nucleic acid molecule encoding a protein of interest or antigenic portion thereof into an avirulent *Salmonella* spp. (see Sambrook et al. 1989). One commonly used method for introducing nucleic acid molecules into a cell is through the use of a plasmid vector. The use of viral vectors such as bacteriophage is another example of a known method for introducing nucleic acid molecules into a cell

(the bacteriophage is used to introduce nucleic acid molecules into a bacterial cell).

The engineered *Salmonella* spp. can be introduced into a host (in which a class I-restricted immune response is desired to the protein of interest) by any methods known in the art, including for example, oral infection or injection.

Materials and Methods

Bacterial strains, cell lines and culture conditions

Bacterial strains used are listed on Table 1 and were grown under conditions that allow expression of components and targets of the invasion-associated type III system (Collazo and Galan 1996). Henle-407 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% bovine calf serum.

Immunofluorescence staining

Henle-407 cells were grown to semi-confluency in 12 mm round glass coverslips that had been previously treated with poly-L-lysine. Cells were infected with different *S. typhimurium* strains in Hank's balanced salt solution (HBSS) for varying times as indicated in each experiment at a multiplicity of infection (m.o.i.) of ~25. When indicated, cytochalasin D was added to the cells 15 minutes before the infection at a concentration of 5 μg/ml. At this concentration, cytochalasin D effectively prevented bacterial internalization. In some experiments, gentamicin was added at a concentration of 100 μg/ml to eliminate extracellular bacteria. After infection, cells were washed three times with HBSS, fixed in 3.7% formaldehyde for 10 min, and permeabilized in 0.2% Triton X-100 in PBS for 2 minutes. After permeabilization, cells were washed three times with phosphate buffered saline containing 3% bovine serum albumin (PBS/BSA) and incubated for 30 min at room temperature with a 1:25 dilution in PBS/BSA of rabbit

polyclonal antisera against SipB, SipC, AvrA, SptP and SopE. After washing three times with PBS/BSA, monolayers were incubated for 30 minutes at room temperature with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG diluted 1:100 in PBS/BSA, washed three times with PBS and stained with DAPI (5 μg/ml). Coverslips were mounted on glass slides with Vectashield mounting medium (Vector Labs, Inc.) and visualized under a Nikon Diaphot 300 fluorescence microscope or under a confocal microscope (Odyssey, Noran Instruments, Middletown WI).

Detection of translocated bacterial proteins in S. typhimurium-infected Henle-407 cells

Semi-confluent Henle-407 cells were grown in 100 mm tissue culture plates and infected with different strains of *S. typhimurium* at an m.o.i. of 50 in 2.5 ml HBSS for 90 min. Prior to the infection, bacterial cultures were centrifuged and resuspended in HBSS to eliminate culture supernatant proteins. After infection, non-adherent bacteria were removed and cells were washed three times with HBSS. The infection supernatant was combined with the material from the washes and centrifuged at 8,000 g for 20 min. The pellet containing non-adherent bacteria was resuspended in 200 μl PBS (non-adherent bacteria fraction). The supernatant was filtered through a 0.45 μm syringe filter (Gelman Sciences, MI) and proteins were precipitated by addition of 10% TCA and subsequent incubation at 4°C for 1 hr (infection medium fraction). Infected cells were incubated for 30 minutes with DMEM containing 10% BCS and 100 μg/ml gentamicin to kill extracellular bacteria and subsequently washed thoroughly with HBSS. Cells were then treated with 30 μg/ml of proteinase K in HBSS for 15 min at 37°C in a CO_2 incubator to eliminate cell-surface associated Sip proteins. After proteinase K treatment, 3 ml of chilled HBSS containing 2mM PMSF were added. Cells detached during the proteinase treatment and were subsequently collected by

low speed centrifugation (600 g for 10 minutes) and lysed in 1 ml of HBSS containing 0.1% Triton X-100 and 1mM PMSF. The cell lysate was transferred to a microcentrifuge tube, treated with DNAse (10 μg/ml) and RNase (10 μg/ml) for 15 min at room temperature and centrifuged at 15,000 g for 10 minutes. The pellet was resuspended in PBS (Triton X-100 insoluble fraction) and the supernatant was filtered through a 0.45 μm syringe filter and proteins were precipitated in the presence of 10% TCA at 4°C (Triton X-100 soluble fraction).

Western blot analysis

Samples were separated in a 10% discontinuous SDS-PAGE, and transferred to nitrocellulose membranes as described (Collazo and Galan 1996). SipB, SipC, AvrA, SptP and SopE proteins were detected by immunoblot analysis using monoclonal antibodies and enhanced chemiluminescence. For quantitation of the Sip proteins in the different cellular fractions, western blots were treated with monoclonal antibodies followed by alkaline phosphatase-labeled anti mouse antibody. Blots were developed by the addition of the fluorescence-emitting Attophos substrate (JBL, San Luis Obispo, CA) and scanned in a Molecular Dynamics Storm unit. The scanned membranes were quantified using the software ImageQuant version 1.1 (Molecular Dynamics) run on a Macintosh Power PC 8100 computer.

EXAMPLE I

SipB, SipC, SptP, AvrA and SopE are translocated into cultured Henle-407 cells in an inv-dependent manner

This experiment was designed to investigate whether infection of cultured intestinal Henle-407 cells with wild-type *S. typhimurium* would result in the translocation of SipB, SipC, SptP, AvrA or SopE which are targets of the invasion-associated type III secretion system (Kaniga et al. 1995a; Kaniga et al. 1995b).

Cultured intestinal Henle-407 cells were infected with the *S. typhimurium* wild-type strain SL1344 or its isogenic mutant strain SB136 which carries a non-polar mutation in *invA*, a gene that encodes an essential component of the invasion-associated type III secretion system (Galan et al. 1992). Two hours after infection, cells were fixed and processed for immunofluorescence staining with antibodies directed to SipB, SipC, AvrA, SptP and SopE as indicated in Materials and Methods.

A fluorescence signal was apparent in samples infected with wild-type *S. typhimurium* and stained with the anti SipB, SipC, AvrA, SptP and SopE antibodies. Henle-407 cells infected with the *invA* mutant strain SB136 exhibited no cell SipC staining. Optical sectioning and confocal microscopy determined that SipC, AvrA, SipB, SopE and SptP were equally distributed throughout the cytoplasm of cells infected with wild-type bacteria. To further demonstrate the translocation of AvrA, SipB, SipC, SptP and SopE into infected cells, cultured Henle-407 cells were infected with *Salmonella typhimurium*. Two hours after infection, a biochemical fractionation of the infected cells was carried out and the presence of these proteins in the different fractions was investigated by Western Blot analysis as indicated in the Materials and Methods. AvrA, SipB, SipC, SptP and SopE were present in the infection media and Triton X-100 soluble and insoluble fractions of Henle-407 infected cells.

These results indicate that SipB, SipC, SptP, SopE and AvrA are translocated into cultured Henle-407 cells and that such a translocation is dependent on the function of the type III protein secretion system encoded at centisome 63 on the *S. typhimurium* chromosome.

EXAMPLE II

Chimeric proteins between SptP, a target of the centisome 63 type III protein secretion system of

Salmonella spp., and heterologous polypeptides are translocated into host cells

Using recombinant DNA technology, a gene encoding a chimeric protein (termed SptP-NP) was constructed by introducing an oligonucleotide encoding a class I restricted epitope (SEQ ID NO:1: IASNENMETMESSTLELRS consisting of residues 365 through 384 of the influenza virus nucleoprotein) at residue 286 of the *Salmonella* SptP protein sequence. The sequence of the resulting chimeric protein (SptP-NP) is as follows (the influenza virus nucleoprotein epitope is depicted underlined and bold):

SEQ ID NO:2:

MLKYEERKLNNLTLSSFSKVGVSNDARLYIAKENTDKAYVAPEKFSSKVLTWLGK
MPLFKNTEVVQKHTENIRVQDQKILQTFLHALTEKYGETAVNDALLMSRINMNKP
LTQRLAVQITECVKAADEGFINLIKSKDNVGVRNAALVIKGGDTKVAEKNNDVGA
ESKQPLLDIALKGLKRTLPQLEQMDGNSLRENFQEMASGNGPLRSLMTNLQNLNK
IPEAKQLNDYVTTLTNIQVGVARFSQWGTCGGEVERWVDKASTHELTQAVKKIHV
IAKELKNVTE**IASNENMETMESSTLELRS**TELEKIEAGAPMPQTMSGPTLGLAR
FAVSSIPINQQTQVKLSDGMPVPVNTLTFDGKPVALAGSYPKNTPDALEAHMKML
LEKECSCLVVLTSEDQMQAKQLPPYFRGSYTFGEVHTNSQKVSSASQGEAIDQYN
MQLSCGEKRYTIPVLHVKNWPDHQPLPSTDQLEYLADRVKNSNQNGAPGRSSSD
KHLPMIHCLGGVGRTGTMAAALVLKDNPHSNLEQVRADFRDSRNNRMLEDASQF
VQLKAMQAQLLMTTAS

A plasmid encoding SptP-NP was introduced into several strains of *Salmonella typhimurium* (listed in Table 1) and the resulting strains tested for their ability to direct the translocation of the chimeric protein into host cells. The translocation of the chimeric proteins was investigated by fluorescence microscopy and biochemical fractionation as described in Materials and Methods. Henle-407 cells were infected with strains of *S. typhimurium* encoding SptP-NP and after two hours of infection, cells were fixed and processed for immunofluorescence staining with a monoclonal antibody capable of detecting the chimeric protein. Infected cells showed intensive SptP-NP staining

throughout the cell cytoplasm. Confocal microscopy established that the chimeric protein was distributed throughout the cell cytoplasm but was absent from the nucleus and plasma membranes of infected cells. These results indicate that *S. typhimurium* can translocate SptP-NP into host cells during infection.

Similar studies were carried out with two additional chimeric proteins. One chimeric protein (termed SptP-LCM) was constructed by introducing an epitope of the lymphocytic coriomeningitis (LCM) virus (SEQ ID NO:3: RSERPQASGVYMGN) at residue 286 of the *Salmonella* SptP protein sequence. The sequence of the resulting chimeric protein (SptP-LCM) is as follows (the LCM epitope is depicted underlined and bold):

SEQ ID NO:4:

MLKYEERKLNNLTLSSFSKVGVSNDARLYIAKENTDKAYVAPEKFSSKVLTWLGK
MPLFKNTEVVQKHTENIRVQDQKILQTFLHALTEKYGETAVNDALLMSRINMNKP
LTQRLAVQITECVKAADEGFINLIKSKDNVGVRNAALVIKGGDTKVAEKNNDVGA
ESKQPLLDIALKGLKRTLPQLEQMDGNSLRENFQEMASGNGPLRSLMTNLQNLNK
IPEAKQLNDYVTTLTNIQVGVARFSQWGTCGGEVERWVDKASTHELTQAVKKIHV
IAKELKNVTE**RSERPQASGVYMGN**TELEKIEAGAPMPQTMSGPTLGLARFAVSS
IPINQQTQVKLSDGMPVPVNTLTFDGKPVALAGSYPKNTPDALEAHMKMLLEKEC
SCLVVLTSEDQMQAKQLPPYFRGSYTFGEVHTNSQKVSSASQGEAIDQYICNCLRG
KAYTSVLHVKNWPDHQPLPSTDQLEYLADRVKNSNQNGAPGASSSDKHLPMIHC
LAGVGRTGTMAGGLVLKDNLIVIWSRYVQIRITT

The other chimeric protein (SptP-NPc) was constructed by fusing the first 173 amino acids of SptP with residues 335 through 498 of the influenza virus nucleoprotein. The sequence of the resulting chimeric protein (SptP-NPc) was as follows (the influenza virus nucleoprotein portion is depicted underlined and bold):

SEQ ID NO:5:

MLKYEERKLNNLTLSSFSKVGVSNDARLYIAKENTDKAYVAPEKFSSKVLTWLGK
MPLFKNTEVVQKHTENIRVQDQKILQTFLHALTEKYGETAVNDALLMSRINMNKP
LTQRLAVQITECVKAADEGFINLIKSKDNVGVRNAALVIKGGDTKVAEKNNDVGA
ESKQPLL**SAAFEDLRVSSFIRGTKVVPRGKLSTRGVQIASNENMETMESST**
LELRSRYWAIRTRSGGNTNQQRASSGQISIQPTFSVQRNLPFDRPTIMAA

FTGNTEGRTSDMRTEIIRLMESARPEDVSFQGRGVFELSDEKAASPIVPSF DMSNEGSYFFGDNAEEYDN

When expressed in various strains of *S. typhimurium* both chimeric proteins (SptP-LCM and SptP-NPc) were translocated into host cells.

EXAMPLE III

Salmonella strains expressing chimeric proteins between SptP and influenza nucleoprotein peptide sequences can present a nucleoprotein epitope via the class I pathway *in vitro*

To examine the ability of the different *Salmonella* strains (SL 1344 and χ3625) expressing SptP-Np and SptP-NPc to present the influenza virus nucleoprotein epitope via the class I presenting pathway *in vitro*, an assay was used based on the induction of IL-2 production in the T cell hybridoma 1192 upon presentation of the NP366-374 peptide in the context of class I D^b molecules by the EL4 murine lymphoma cell line. EL4 cells (~$6x10^7$), grown in Dulbecco's modified MEM with 10% fetal bovine serum, 10 mM HEPES, $5x10^{-5}$ M 2-mercaptoethanol and without antibiotics (DMEM-10), were alternatively infected with $6x10^9$ c.f.u. of the different strains of *S. typhimurium* (constructed as described in Example II) expressing either SptP-NP or SptP-NP. Infections were carried out as described (Kaniga et al. 1996). After infection, non-internalized or adhered bacteria were removed by washing, and cells further incubated in the presence of 100 μg/ml of gentamicin for 1 hour. A volume of 0.1 ml of DMEM-10 containing $2x10^5$ EL4 cells infected with *Salmonella* were distributed into each well of 96-well plates in quadruplicate. To each well $1x10^5$ 1192 T cell hybridoma effector cells (clones 12.164 or 7.9.3.28) in 0.1 ml of DMEM-10 were added. Plates were then incubated for 10 hours at 37°C, 100 μl of supernatant fluid removed from each well, divided in two aliquots, placed in individual wells of a 96 well plate and stored at -20°C for

subsequent IL-2 quantitation. The presence of IL-2 in the cell culture media aliquots was determined using the IL-2-dependent cell line CTLL-2 (ATCC TIB-214) as follows. To the thawed plates containing the test samples (EL4 + 1192 supernatants) $5x10^4$ CTLL-2 cells in 100 µl of DMEM-10 were added and the plates incubated for 16-18 hours at 37°C. CTLL-2 proliferation in response to cytokines was measured by the MTT dye reduction assay. The reduction of the yellow MTT to a blue formazan is proportional to the number and metabolic rate of the CTLL-2 cells. To this end, 20 µl of a 5mg/ml sterile solution of MTT was added to all wells and the plates incubated for 6 hours at 37°C. The reduced MTT formazan was solubilized with acid isopropanol and the absorbance of each well was measured in an ELISA plate reader equipped with a 570 nm filter. The response was compared to a standard curve prepared by adding purified recombinant IL-2 to parallel cultures of CTLL-2 cells.

Influenza virus PR8-infected EL4 cells (multiplicity of infection of 10) and influenza virus nucleoprotein peptide NP366-374 served as positive controls. EL4 cells infected with the same *S. typhimurium* strains expressing the influenza virus nucleoprotein not fused to the SptP protein (NP) and therefore unable to be delivered into host cells served as negative controls. The results are shown in Table 2.

EXAMPLE IV

Additional chimeric proteins are translocated into host cells

A chimeric form of SptP that carries a Class-I restricted epitope consisting of residues 366 through 374 from the influenza virus nucleoprotein ($IVNP_{366-374}$) found to be immunodominant in mice of the $H-2^b$ haplotype (Vitiello et al. 1996: Rotzschke et al. 1990) was constructed. The epitope was introduced at a permissive site of SptP located between the two predicted

independent domains of this protein (Fig. 1) (Kaniga et al. 1996; Fu and Galan 1998).

Plasmid pSB762, which encodes a chimeric protein consisting of SptP fused to a region of the influenza virus NP comprising residues 366 through 374 (SEQ ID NO:6: ASNENMETM) of the influenza virus nucleoprotein, was constructed by inserting a double stranded oligonucleotide (SEQ ID NO:7: 5'-AAATTGCTTCCAATGAAAACATGGAGACTATGGAATCAAGTACCCTTGAACTGAGAA GCA-3') into the unique *Pvu*II site of *sptP*. A double stranded oligonucleotide (SEQ ID NO:8: 5'-AACGTAGCGAACGGCCGCAAGCATCAGGCGTTTATATGGGAAATA-3') was inserted into the unique *Pvu*II site of *sptP* to construct plasmid pSB775 which encodes a protein fusion between SptP and residues 118 through 126 of the LCMV nucleoprotein. Both plasmids are derived from the low copy plasmid pWSK-II (Wang and Kushner 1991). Random expression of the chimeric protein is driven by the *plac* promoter. Plasmid pSB776, which encodes a fusion between the first 179 amino acids of InvJ and residues 335 through 498 of the influenza virus nucleoprotein was constructed by polymerase chain reaction. This plasmid is derived from the low copy plasmid pYA292 (Galan et al. 1990) and expression of the chimeric protein is driven by the *plac* promoter. Strain SB824 was constructed by introducing the *sptP*::kan mutant allele from strain SB237 (Kaniga et al. 1996; Fu and Galan 1998) into the Δ*aroA* strain SL3261 (Hoiseth and Stocker 1981) via P22HT*int* transduction.

The chimeric SptP-IVNP$_{366-374}$ protein was secreted into the culture supernatant of both wild-type *S. typhimurium* and the isogenic avirulent *aroA sptP* mutant strain SB824 at levels indistinguishable from those of wild-type SptP. Furthermore, both strains efficiently delivered Spt-IVNP$_{366-374}$ into the cytosol of infected cultured epithelial cells. In contrast, and as expected, the isogenic *S. typhimurium sipD* mutant strain SB221 failed to

translocate the chimeric protein into the host-cell cytosol although it was able to secrete SptP-IVNP$_{366-374}$ to the infection medium. SipD is essential for type III protein translocation into host cells although it is completely dispensable for protein secretion (Kaniga et al. 1996; Fu and Gálan 1998; Kaniga et al. 1995). Similar results were obtained when a different epitope derived from the lymphocytic choriomeningitis virus nucleoprotein (LCMVNP$_{118-126}$) was introduced at the same site of SptP. These results indicate that SptP can serve as a "molecular courier" to deliver foreign peptides of immunological interest to the cytoplasm of target cells.

EXAMPLE V

Salmonella strains expressing the additional chimeric proteins can present a nucleoprotein epitope via the class I pathway *in vitro*

The ability of wild type, *aroA* and *aroA sptP S. typhimurium* expressing the chimeric protein SptP-IVNP$_{366-374}$ to deliver the influenza nucleoprotein (NP) epitope to a Class I-restricted antigen presenting pathway was then examined. Murine RMA thymoma cells (H-2^b) were infected with the different *S. typhimurium* strains and the ability of the infected cells to present the influenza NP epitope to the Class I-restricted T-cell hybridoma 12.164 was assessed by measuring its IL-2 secretory response (Deckhut et al. 1993; Ljunggren and Karre 1985).

RMA or RMA-S cells (C57/BL/6J mouse lymphoma), cultured without antibiotics in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), were used as antigen presenting cells (APC) (Deckhut et al. 1993; Ljunggren and Karre 1985). APC cells were infected with *S. typhimurium* strains grown as previously described (Collazo and Galan 1996). Approximately 3 X 10^8 bacterial colony forming units were used to infect 10^7 APC cells in a 3 ml volume of DMEM at 37°C for 2 hrs. Control APC cells were

prepared by infection with $10^{7.5}$ plaque forming units (PFU) of influenza A virus (strain A/PR/8/34) or incubation in 1 μM $IVNP_{366-374}$ synthetic peptide (sequence: SEQ ID NO:6: ASNENMETM) for 2 hrs. APC cells subjected to the different procedures were subsequently washed and incubated for 1 hr in DMEM containing 100 μg/ml gentamicin. APC cells (2 X 10^6/ml) suspended in DMEM supplemented with FBS and gentamicin (50 μg/ml) were distributed in quadruplicate 100 μl cultures in 96-well plates. Influenza NP-specific T-cell hybridoma 12.164 cells (2 X 10^5/100 μl/well) were added to the treated RMA or RMA-S cultures (Deckhut et al. 1993; Ljunggren and Karre 1985). Cultures were incubated for 44 hrs at 37°C and the culture medium harvested to determine the IL-2 concentration in the supernatant by a capture ELISA assay using rat anti-mouse IL-2 monoclonal antibodies (Pharmingen).

As shown in Fig. 2, the RMA thymoma cells infected with *S. typhimurium* strains expressing SptP-$IVNP_{366-374}$ were efficiently recognized by the epitope-specific T-cell hybridoma. Antigen presentation was strictly dependent on the cytosolic delivery of the epitope by the *S. typhimurium* type III system, as RMA cells infected with a *sipD*$^-$ mutant strain, which is capable of secreting SptP-$IVNP_{366-374}$ but is unable to deliver it into the cell cytosol, failed to stimulate the T-cell hybridoma (Fig. 2). Furthermore, *S. typhimurium* strains expressing the carboxy terminal half of the influenza virus NP fused to InvJ (InvJ-$IVNP_{335-498}$), a protein substrate of the type III system that is secreted but it is not translocated into host cells (Collazo and Galan 1996; Collazo et al. 1995), failed to present antigen in infected RMA cells (Fig. 2). These results indicate that *S. typhimurium* is capable of delivering foreign epitopes to the Class I antigen presenting pathway via proteins translocated by the type III secretion system.

Peptides delivered to the cytosol by *S. typhimurium* required a functional peptide transporter system (TAP) for their transfer to the endoplasmic reticulum and loading by Class I molecules since TAP2-defective RMA-S mutant cells (Attaya et al. 1992) infected by *S. typhimurium* SptP-INVP$_{366-374}$ were markedly impaired in their capacity to stimulate the T-cell hybridoma when compared to the wild-type RMA cells (Fig. 3). This result provides independent evidence that peptides displayed at the RMA cell surface by Class-I molecules occupied a cytosolic compartment prior to their transfer into the endoplasmic reticulum.

EXAMPLE VI

Salmonella strains expressing the additional chimeric proteins can induce protective Class I-restricted cytotoxic T-lymphocytes *in vivo*

The potential of avirulent *S. typhimurium* strains expressing SptP-IVNP$_{366-374}$ to induce cytotoxic T-lymphocytes (CTL) in vivo was then examined. C57/BL/6J mice orally inoculated with an avirulent *S. typhimurium sptP aroA* mutant strain expressing SptP-IVNP$_{366-374}$ developed CTL precursors which, upon re-stimulation, lysed target cells infected with influenza virus or loaded with the synthetic peptide NP$_{366-374}$ (Fig. 4).

Cytotoxic T lymphocytes (CTL) specific for influenza or LCMV were quantitated as described elsewhere (Wunderlich and Shearer 1991). Briefly, spleen cells from two or three mice were pooled and re-stimulated *in vitro* for 5 days by co-culture with irradiated syngeneic splenocytes infected with A/PR/8/34 influenza virus at a multiplicity of infection of 3, or treated with 1 μM of a synthetic peptide with sequence derived from the LCMVNP$_{118-126}$ (sequence: SEQ ID NO:9: RPQASGVYM). EL-4 (H-2^b) and P815 (H-2^d) target cells were prepared by incubation with 1 μM IVNP$_{366-374}$, or LCMVNP$_{118-126}$ peptides and followed by labeling with 0.15 mCi of [^{51}Cr]-Na chromate. To set

cultures with a range of effector to target (E:T) ratios, appropriate numbers of effector cells were combined with constant numbers of target cells in quadruplicate cultures using 96-well plates. Target lysis was assessed by ^{51}Cr release after co-cultivation for 4 hours. Target lysis was calculated from the standard formula: [(Experimental release - Spontaneous release) / (Maximum release - Spontaneous release)] X 100. Maximum release was determined by Triton X-100 cell lysis. Spontaneous release was always <10% of maximum release. Data are presented as % specific lysis, with the % lysis of unsensitized target cells subtracted from the % lysis of cognate peptide sensitized target cells.

In contrast and consistent with the in vitro antigen presentation results, mice inoculated with *Salmonella* expressing either the irrelevant epitope SptP-LCMVNP$_{118-126}$ or non-translocatable InvJ-IVNP$_{335-498}$ failed to stimulate CTL directed to influenza virus epitopes (Fig. 4). Thus, cytoplasmic targeting of the type III system hybrid substrates dictates the outcome of the Class I-restricted response to a foreign epitope: pronounced CTL responses were induced by the *S. typhimurium* mutant strain expressing SptP-IVNP$_{366-374}$ but not by the strain expressing the non-translocated InvJ-IVNP$_{335-498}$ chimeric protein.

To assess the ability of the type III-mediated *S. typhimurium* antigen delivery system to induce protective Class I-restricted CTL, murine lymphocytic choriomeningitis virus (LCMV) infection was chosen because it is the most reliable model to assess the role of CTL in protection against viral disease (Klavinskis et al. 1989; Whitton et al. 1989). In this model, intracerebral inoculation with LCMV results in a lethal choriomeningitis which can be prevented by a single clonal population of LCMV-specific CTL (Klavinskis et al. 1989; Whitton et al. 1989). BALB/c mice immunized with the *aroA sptP S. typhimurium* mutant strain expressing SptP-LCMVNP$_{118-126}$ were completely protected against lethal

intracerebral challenge with a virulent strain of LCMV (Fig. 5).

C57BL/6J or BALB/c mice were fasted for 6 hrs and given 100 μl of 10% bicarbonate orally one hour prior to intragastric inoculation with 1-2 X 10^8 CFU of appropriate live *S. typhimurium* strains grown as described (Collazo and Galan 1996). Intragastric inoculation was repeated at 4- and 6- weeks after the initial inoculation. Spleens were removed two weeks after the last bacterial inoculation to evaluate CTL activity in three mice from each group. To determine protective immunity, groups of 6-8 vaccinated mice were inoculated intracerebrally with 10 LD_{50} of LCMV strain Armstrong. For control purposes, mice were inoculated intraperitoneally with $10^{7.5}$ PFU of influenza virus A/PR/8/34, 10^6 PFU of vaccinia influenza NP or 10^4 PFU of LCMV strain Armstrong four weeks prior to immunity assessment.

In contrast, mice immunized with *S. typhimurium aroA* mutant strain expressing an irrelevant epitope (SptP-$IVNP_{366-374}$) succumbed to the same challenge (Fig. 5). Consistent with the hypothesis that protection was mediated by an H-2 restricted immune response, the same *S. typhimurium aroA* mutant strain expressing SptP-$LCMVNP_{118-126}$ failed to protect C57/BL/6J mice against an identical challenge with LCMV. Furthermore, protection required cytosolic delivery of the epitope by the type III secretion system as vaccination with a *S. typhimurium sipD*⁻ strain, defective in translocation but not in secretion of SptP-$LCMVNP_{118-126}$ failed to protect mice against lethal LCMV infection (Fig. 5). Induction of protective immunity in mice by *Salmonella* vaccination was correlated with the presence of LCMV-specific CTL (Fig. 6).

These results show that delivery of epitopes through the *S. typhimurium* type III secretion system results in efficient stimulation of Class-I restricted protective antiviral immune responses. This approach expands the

use of *S. typhimurium* as a carrier of heterologous antigens to vaccinate against infections in which this type of response is crucial for protection (Hackett 1993; Karen et al. 1997). In addition, avirulent *S. typhimurium* expressing tumor-specific antigens allow the use of this system for the treatment of neoplastic diseases by induction of cancer cell-specific Class I-restricted CTL (Schmidt et al. 1997; Ossevoort et al. 1995; Schlom et al. 1996).

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1. Bacterial strains used in this study.

Strains	Relevant Genotype	Reference or source
S. typhimurium		
SL1344	*rpsL hisG*	(Hoiseth and Stocker 1981)
SB136	*rpsL hisG invA::aphT*	(Galan et al. 1992)
SB225	*rpsL hisG sipA::aphT*	(Kaniga et al. 1995)
SB169	*rpsL hisG sipB::aphT*	(Kaniga et al. 1995)
SB220	*rpsL hisG sipC::aphT*	(Kaniga et al. 1995)
SB221	*rpsL hisG sipD::aphT*	(Kaniga et al. 1995)
SB237	*rpsL hisG sptP::aphT*	(Kaniga et al. 1996)
χ3625	*rpsL hisG aroA::Tn10*	(Hoiseth and Stocker 1981)

Table 2. Class I-restricted antigen presentation following challenge of antigen presenting cells with *S. typhimurium* expressing SptP-NP.

Challenge	Hybridoma	IL-2 (pg)
PR8	12.164	800±70
PR8	7.9.3.28	4167±320
NP366-374	12.164	213±10
NP366-374	7.9.3.28	1250±80
SL1344 (SptP-NP)	12.164	172±10
SL1344 (SptP-NP)	7.9.3.28	300±18
SL1344 (NP)	12.164	<20*
SL1344 (NP)	7.9.3.28	<20*
χ3625 (SptP-NP)	12.164	314±30
χ3625 (NP)	12.164	<20*

*This values were considered negative as they were below the detection range of the assay

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Research Foundation of State University of New York

(ii) TITLE OF INVENTION: ANTIGEN DELIVERY SYSTEM

(iii) NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Leu Lys Tyr Glu Glu Arg Lys Leu Asn Asn Leu Thr Leu Ser Ser
1 5 10 15

Phe Ser Lys Val Gly Val Ser Asn Asp Ala Arg Leu Tyr Ile Ala Lys
20 25 30

Glu Asn Thr Asp Lys Ala Tyr Val Ala Pro Glu Lys Phe Ser Ser Lys
35 40 45

Val Leu Thr Trp Leu Gly Lys Met Pro Leu Phe Lys Asn Thr Glu Val
50 55 60

Val Gln Lys His Thr Glu Asn Ile Arg Val Gln Asp Gln Lys Ile Leu
65 70 75 80

Gln Thr Phe Leu His Ala Leu Thr Glu Lys Tyr Gly Glu Thr Ala Val
85 90 95

Asn Asp Ala Leu Leu Met Ser Arg Ile Asn Met Asn Lys Pro Leu Thr
100 105 110

Gln Arg Leu Ala Val Gln Ile Thr Glu Cys Val Lys Ala Ala Asp Glu
115 120 125

Gly Phe Ile Asn Leu Ile Lys Ser Lys Asp Asn Val Gly Val Arg Asn
130 135 140

Ala Ala Leu Val Ile Lys Gly Gly Asp Thr Lys Val Ala Glu Lys Asn
145 150 155 160

Asn Asp Val Gly Ala Glu Ser Lys Gln Pro Leu Leu Ser Ala Ala Phe
165 170 175

Glu Asp Leu Arg Val Ser Ser Phe Ile Arg Gly Thr Lys Val Val Pro
180 185 190

Arg Gly Lys Leu Ser Thr Arg Gly Val Gln Ile Ala Ser Asn Glu Asn
195 200 205

Met Glu Thr Met Glu Ser Ser Thr Leu Glu Leu Arg Ser Arg Tyr Trp
210 215 220

Ala Ile Arg Thr Arg Ser Gly Gly Asn Thr Asn Gln Gln Arg Ala Ser
225 230 235 240

Ser Gly Gln Ile Ser Ile Gln Pro Thr Phe Ser Val Gln Arg Asn Leu
245 250 255

Pro Phe Asp Arg Pro Thr Ile Met Ala Ala Phe Thr Gly Asn Thr Glu
260 265 270

Gly Arg Thr Ser Asp Met Arg Thr Glu Ile Ile Arg Leu Met Glu Ser
275 280 285

Ala Arg Pro Glu Asp Val Ser Phe Gln Gly Arg Gly Val Phe Glu Leu
290 295 300

Ser Asp Glu Lys Ala Ala Ser Pro Ile Val Pro Ser Phe Asp Met Ser
305 310 315 320

Asn Glu Gly Ser Tyr Phe Phe Gly Asp Asn Ala Glu Glu Tyr Asp Asn
325 330 335

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Ile Ala Ser Asn Glu Asn Met Glu Thr Met Glu Ser Ser Thr Leu Glu
1               5                  10                  15

Leu Arg Ser
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Leu Lys Tyr Glu Glu Arg Lys Leu Asn Asn Leu Thr Leu Ser Ser
1               5                  10                  15

Phe Ser Lys Val Gly Val Ser Asn Asp Ala Arg Leu Tyr Ile Ala Lys
            20                  25                  30

Glu Asn Thr Asp Lys Ala Tyr Val Ala Pro Glu Lys Phe Ser Ser Lys
        35                  40                  45

Val Leu Thr Trp Leu Gly Lys Met Pro Leu Phe Lys Asn Thr Glu Val
    50                  55                  60

Val Gln Lys His Thr Glu Asn Ile Arg Val Gln Asp Gln Lys Ile Leu
65                  70                  75                  80

Gln Thr Phe Leu His Ala Leu Thr Glu Lys Tyr Gly Glu Thr Ala Val
                85                  90                  95

Asn Asp Ala Leu Leu Met Ser Arg Ile Asn Met Asn Lys Pro Leu Thr
            100                 105                 110

Gln Arg Leu Ala Val Gln Ile Thr Glu Cys Val Lys Ala Ala Asp Glu
        115                 120                 125

Gly Phe Ile Asn Leu Ile Lys Ser Lys Asp Asn Val Gly Val Arg Asn
    130                 135                 140

Ala Ala Leu Val Ile Lys Gly Gly Asp Thr Lys Val Ala Glu Lys Asn
145                 150                 155                 160

Asn Asp Val Gly Ala Glu Ser Lys Gln Pro Leu Leu Asp Ile Ala Leu
                165                 170                 175

Lys Gly Leu Lys Arg Thr Leu Pro Gln Leu Glu Gln Met Asp Gly Asn
            180                 185                 190
```

Ser Leu Arg Glu Asn Phe Gln Glu Met Ala Ser Gly Asn Gly Pro Leu
195 200 205

Arg Ser Leu Met Thr Asn Leu Gln Asn Leu Asn Lys Ile Pro Glu Ala
210 215 220

Lys Gln Leu Asn Asp Tyr Val Thr Thr Leu Thr Asn Ile Gln Val Gly
225 230 235 240

Val Ala Arg Phe Ser Gln Trp Gly Thr Cys Gly Gly Glu Val Glu Arg
245 250 255

Trp Val Asp Lys Ala Ser Thr His Glu Leu Thr Gln Ala Val Lys Lys
260 265 270

Ile His Val Xaa Ile Ala Lys Glu Leu Lys Asn Val Thr Glu Ile Ala
275 280 285

Ser Asn Glu Asn Met Glu Thr Met Glu Ser Ser Thr Leu Glu Leu Arg
290 295 300

Ser Thr Glu Leu Glu Lys Ile Glu Ala Gly Ala Pro Met Pro Gln Thr
305 310 315 320

Met Ser Gly Pro Thr Leu Gly Leu Ala Arg Phe Ala Val Ser Ser Ile
325 330 335

Pro Ile Asn Gln Gln Thr Gln Val Lys Leu Ser Asp Gly Met Pro Val
340 345 350

Pro Val Asn Thr Leu Thr Phe Asp Gly Lys Pro Val Ala Leu Ala Gly
355 360 365

Ser Tyr Pro Lys Asn Thr Pro Asp Ala Leu Glu Ala His Met Lys Met
370 375 380

Leu Leu Glu Lys Glu Cys Ser Cys Leu Val Val Leu Thr Ser Glu Asp
385 390 395 400

Gln Met Gln Ala Lys Gln Leu Pro Pro Tyr Phe Arg Gly Ser Tyr Thr
405 410 415

Phe Gly Glu Val His Thr Asn Ser Gln Lys Val Ser Ser Ala Ser Gln
420 425 430

Gly Glu Ala Ile Asp Gln Tyr Asn Met Gln Leu Ser Cys Gly Glu Lys
435 440 445

Arg Tyr Thr Ile Pro Val Leu His Val Lys Asn Trp Pro Asp His Gln
450 455 460

Pro Leu Pro Ser Thr Asp Gln Leu Glu Tyr Leu Ala Asp Arg Val Lys
465 470 475 480

Asn Ser Asn Gln Asn Gly Ala Pro Gly Arg Ser Ser Ser Asp Lys His
485 490 495

Leu Pro Met Ile His Cys Leu Gly Gly Val Gly Arg Thr Gly Thr Met
500 505 510

Ala Ala Ala Leu Val Leu Lys Asp Asn Pro His Ser Asn Leu Glu Gln
515 520 525

Val Arg Ala Asp Phe Arg Asp Ser Arg Asn Asn Arg Met Leu Glu Asp
530 535 540

Ala Ser Gln Phe Val Gln Leu Lys Ala Met Gln Ala Gln Leu Leu Met
545 550 555 560

Thr Thr Ala Ser

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Ser Glu Arg Pro Gln Ala Ser Gly Val Tyr Met Gly Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 529 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Leu Lys Tyr Glu Glu Arg Lys Leu Asn Asn Leu Thr Leu Ser Ser
1 5 10 15

Phe Ser Lys Val Gly Val Ser Asn Asp Ala Arg Leu Tyr Ile Ala Lys
20 25 30

Glu Asn Thr Asp Lys Ala Tyr Val Ala Pro Glu Lys Phe Ser Ser Lys
35 40 45

Val Leu Thr Trp Leu Gly Lys Met Pro Leu Phe Lys Asn Thr Glu Val
50 55 60

Val Gln Lys His Thr Glu Asn Ile Arg Val Gln Asp Gln Lys Ile Leu
65 70 75 80

Gln Thr Phe Leu His Ala Leu Thr Glu Lys Tyr Gly Glu Thr Ala Val
85 90 95

Asn Asp Ala Leu Leu Met Ser Arg Ile Asn Met Asn Lys Pro Leu Thr
100 105 110

Gln Arg Leu Ala Val Gln Ile Thr Glu Cys Val Lys Ala Ala Asp Glu
115 120 125

Gly Phe Ile Asn Leu Ile Lys Ser Lys Asp Asn Val Gly Val Arg Asn
130 135 140

```
Ala Ala Leu Val Ile Lys Gly Gly Asp Thr Lys Val Ala Glu Lys Asn
145                 150                 155                 160

Asn Asp Val Gly Ala Glu Ser Lys Gln Pro Leu Leu Asp Ile Ala Leu
                165                 170                 175

Lys Gly Leu Lys Arg Thr Leu Pro Gln Leu Glu Gln Met Asp Gly Asn
            180                 185                 190

Ser Leu Arg Glu Asn Phe Gln Glu Met Ala Ser Gly Asn Gly Pro Leu
        195                 200                 205

Arg Ser Leu Met Thr Asn Leu Gln Asn Leu Asn Lys Ile Pro Glu Ala
    210                 215                 220

Lys Gln Leu Asn Asp Tyr Val Thr Thr Leu Thr Asn Ile Gln Val Gly
225                 230                 235                 240

Val Ala Arg Phe Ser Gln Trp Gly Thr Cys Gly Gly Glu Val Glu Arg
                245                 250                 255

Trp Val Asp Lys Ala Ser Thr His Glu Leu Thr Gln Ala Val Lys Lys
            260                 265                 270

Ile His Val Xaa Ile Ala Lys Glu Leu Lys Asn Val Thr Glu Arg Ser
        275                 280                 285

Glu Arg Pro Gln Ala Ser Gly Val Tyr Met Gly Asn Thr Glu Leu Glu
    290                 295                 300

Lys Ile Glu Ala Gly Ala Pro Met Pro Gln Thr Met Ser Gly Pro Thr
305                 310                 315                 320

Leu Gly Leu Ala Arg Phe Ala Val Ser Ser Ile Pro Ile Asn Gln Gln
                325                 330                 335

Thr Gln Val Lys Leu Ser Asp Gly Met Pro Val Pro Val Asn Thr Leu
            340                 345                 350

Thr Phe Asp Gly Lys Pro Val Ala Leu Ala Gly Ser Tyr Pro Lys Asn
        355                 360                 365

Thr Pro Asp Ala Leu Glu Ala His Met Lys Met Leu Leu Glu Lys Glu
    370                 375                 380

Cys Ser Cys Leu Val Val Leu Thr Ser Glu Asp Gln Met Gln Ala Lys
385                 390                 395                 400

Gln Leu Pro Pro Tyr Phe Arg Gly Ser Tyr Thr Phe Gly Glu Val His
                405                 410                 415

Thr Asn Ser Gln Lys Val Ser Ser Ala Ser Gln Gly Glu Ala Ile Asp
            420                 425                 430

Gln Tyr Ile Cys Asn Cys Leu Arg Gly Lys Ala Tyr Thr Ser Val Leu
        435                 440                 445

His Val Lys Asn Trp Pro Asp His Gln Pro Leu Pro Ser Thr Asp Gln
    450                 455                 460

Leu Glu Tyr Leu Ala Asp Arg Val Lys Asn Ser Asn Gln Asn Gly Ala
465                 470                 475                 480

Pro Gly Ala Ser Ser Ser Asp Lys His Leu Pro Met Ile His Cys Leu
                485                 490                 495
```

Ala Gly Val Gly Arg Thr Gly Thr Met Ala Gly Gly Leu Val Leu Lys
500 505 510

Asp Asn Leu Ile Val Ile Trp Ser Arg Tyr Val Gln Ile Arg Ile Thr
515 520 525

Thr

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Ser Asn Glu Asn Met Glu Thr Met
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAATTGCTTC CAATGAAAAC ATGGAGACTA TGGAATCAAG TACCCTTGAA CTGAGAAGCA 60

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AACGTAGCGA ACGGCCGCAA GCATCAGGCG TTTATATGGG AAATA 45

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Pro Gln Ala Ser Gly Val Tyr Met
1 5

What Is Claimed Is:

1. A method of stimulating a class I-restricted immune response to a protein of interest or antigenic portion thereof in a host, said method comprising:

introducing a nucleic acid molecule encoding a protein of interest or antigenic portion thereof into an avirulent *Salmonella* spp., the nucleic acid molecule being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and

introducing the resulting *Salmonella* spp. into a host, wherein the resulting *Salmonella* spp. injects the chimeric protein into the cytosol of cells of the host thereby stimulating a class I-restricted immune response to the protein of interest or antigenic portion thereof in the host.

2. The method of claim 1 wherein the protein of interest comprises a microbial protein.

3. The method of claim 1 wherein the injectable protein is an injectable protein of *Salmonella*.

4. The method of claim 1 wherein the injectable protein is an injectable protein of *Yersinia*.

5. The method of claim 1 wherein the injectable protein is an injectable protein of *Shigella*.

6. The method of claim 1 wherein the injectable protein is a target of a type III secretion system of a bacteria.

7. A protein delivery vehicle comprising:

an avirulent *Salmonella* spp. encoding a chimeric protein, the chimeric protein comprising a protein of interest or an antigenic portion thereof and an injectable protein which is a target of a type III secretion system or an injectable portion thereof.

8. The protein delivery vehicle of claim 7 wherein the protein of interest comprises a microbial protein.

9. The protein delivery vehicle of claim 7 wherein the injectable protein is an injectable protein of *Salmonella*.

10. The protein delivery vehicle of claim 7 wherein the injectable protein is an injectable protein of *Yersinia*.

11. The protein delivery vehicle of claim 7 wherein the injectable protein is an injectable protein of *Shigella*.

12. The protein delivery vehicle of claim 7 wherein the injectable protein is a target of a type III secretion system of a bacteria.

13. A host into which the protein delivery vehicle of claim 7 has been introduced.

14. A chimeric protein comprising:

a first amino acid sequence of an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and

a second amino acid sequence of a protein of interest or antigenic portion thereof introduced into the first amino acid sequence of the injectable protein or injectable portion thereof.

15. The chimeric protein of claim 14 wherein the protein of interest comprises a microbial protein.

16. The chimeric protein of claim 14 wherein the injectable protein is an injectable protein of *Salmonella*.

17. The chimeric protein of claim 14 wherein the injectable protein is an injectable protein of *Yersinia*.

18. The chimeric protein of claim 14 wherein the injectable protein is an injectable protein of *Shigella*.

19. The chimeric protein of claim 14 wherein the injectable protein is a target of a type III secretion system of a bacteria.

20. A chimeric nucleic acid molecule comprising:

a first nucleic acid sequence encoding an injectable protein which is a target of a type III secretion system or an injectable portion thereof; and

a second nucleic acid sequence encoding a protein of interest or antigenic portion thereof introduced into the first nucleic acid sequence of the injectable protein or injectable portion thereof, the second nucleic acid sequence being introduced so as to encode a chimeric protein comprising the protein of interest or antigenic portion thereof and the injectable protein or injectable portion thereof.

21. The chimeric nucleic acid molecule of claim 20 wherein the protein of interest comprises a microbial protein.

22. The chimeric nucleic acid molecule of claim 20 wherein the injectable protein is a target of any type III secretion system of any bacteria.

23. The chimeric nucleic acid molecule of claim 20 wherein the injectable protein is an injectable protein of *Yersinia*.

24. The chimeric nucleic acid molecule of claim 20 wherein the injectable protein is an injectable protein of *Shigella*.

25. The chimeric nucleic acid molecule of claim 20 wherein the injectable protein is a target of a type III secretion system of a bacteria.

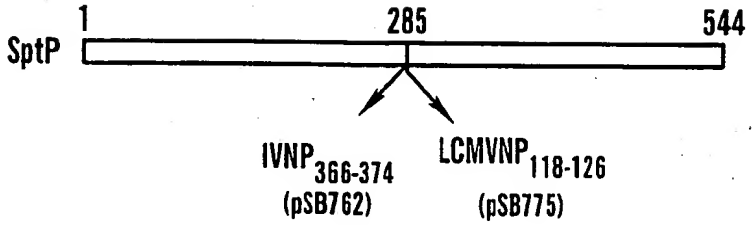
1
285
544
SptP
IVNP366-374
(pSB762)
LCMVNP118-126
(pSB775)

FIG. 1

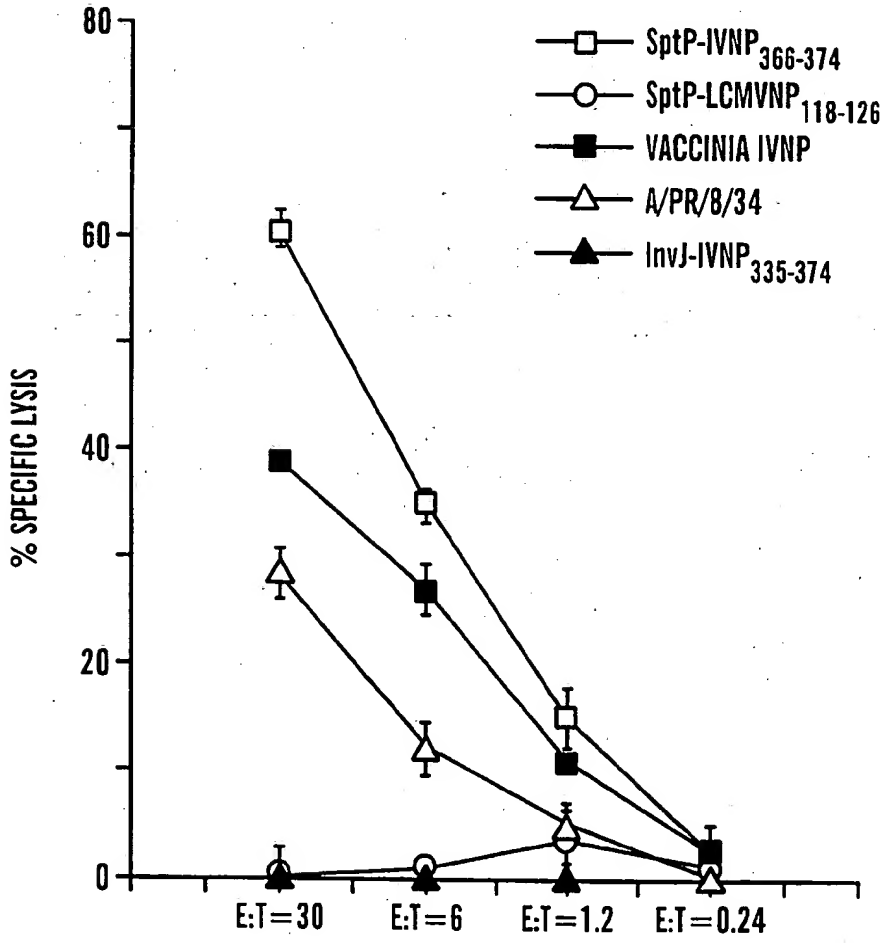
80
60
40
20
0
% SPECIFIC LYSIS
SptP-IVNP366-374
SptP-LCMVNP118-126
VACCINIA IVNP
A/PR/8/34
InvJ-IVNP335-374
E:T=30
E:T=6
E:T=1.2
E:T=0.24

FIG. 4

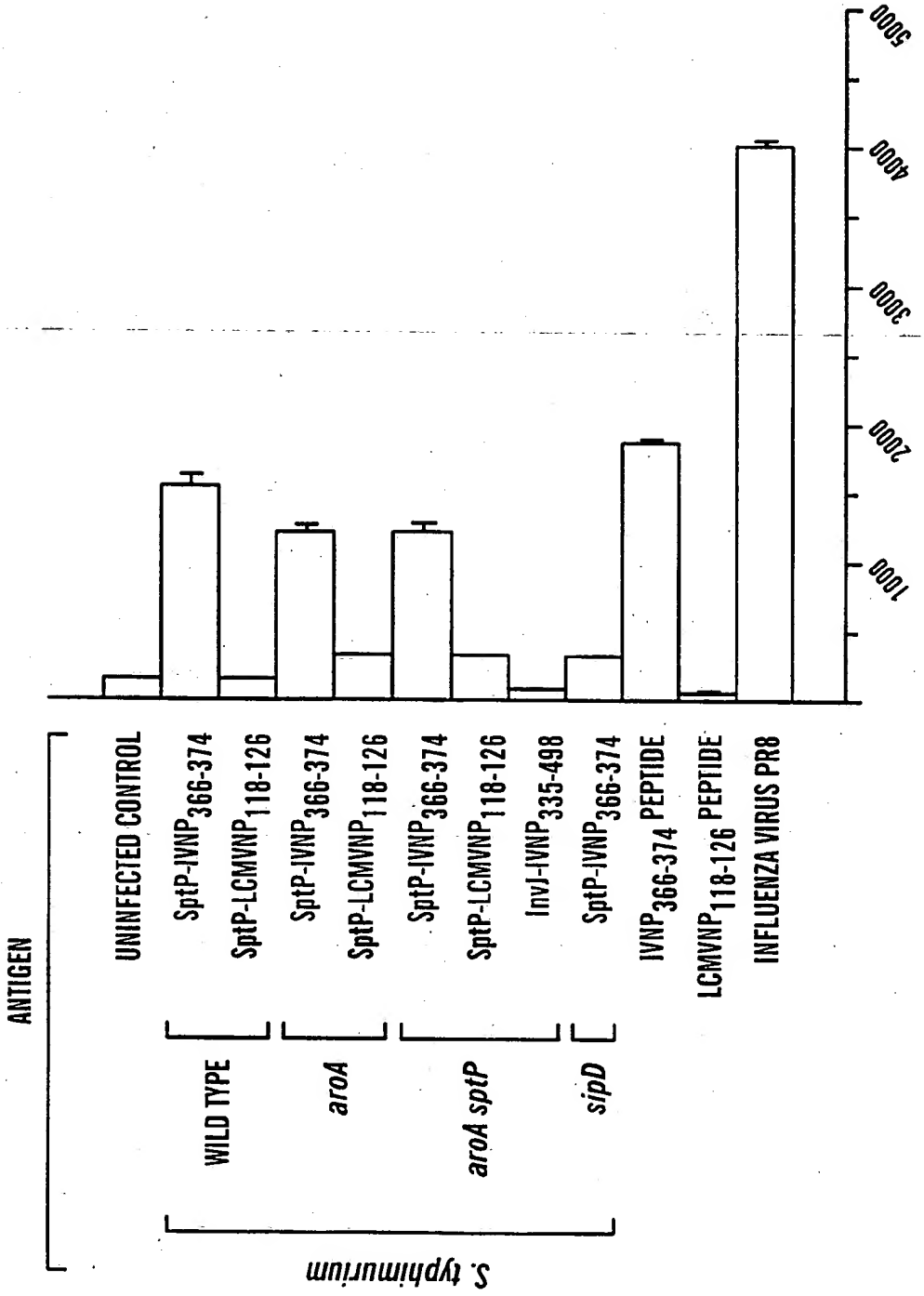
IL2 SECRETION (pg)
1000
2000
3000
4000
5000
ANTIGEN
S. typhimurium
WILD TYPE
aroA
aroA sptP
sipD
UNINFECTED CONTROL
SptP-IVNP366-374
SptP-LCMVNP118-126
SptP-IVNP366-374
SptP-LCMVNP118-126
SptP-IVNP366-374
SptP-LCMVNP118-126
InvJ-IVNP335-498
SptP-IVNP366-374
IVNP366-374 PEPTIDE
LCMVNP118-126 PEPTIDE
INFLUENZA VIRUS PR8

FIG. 2

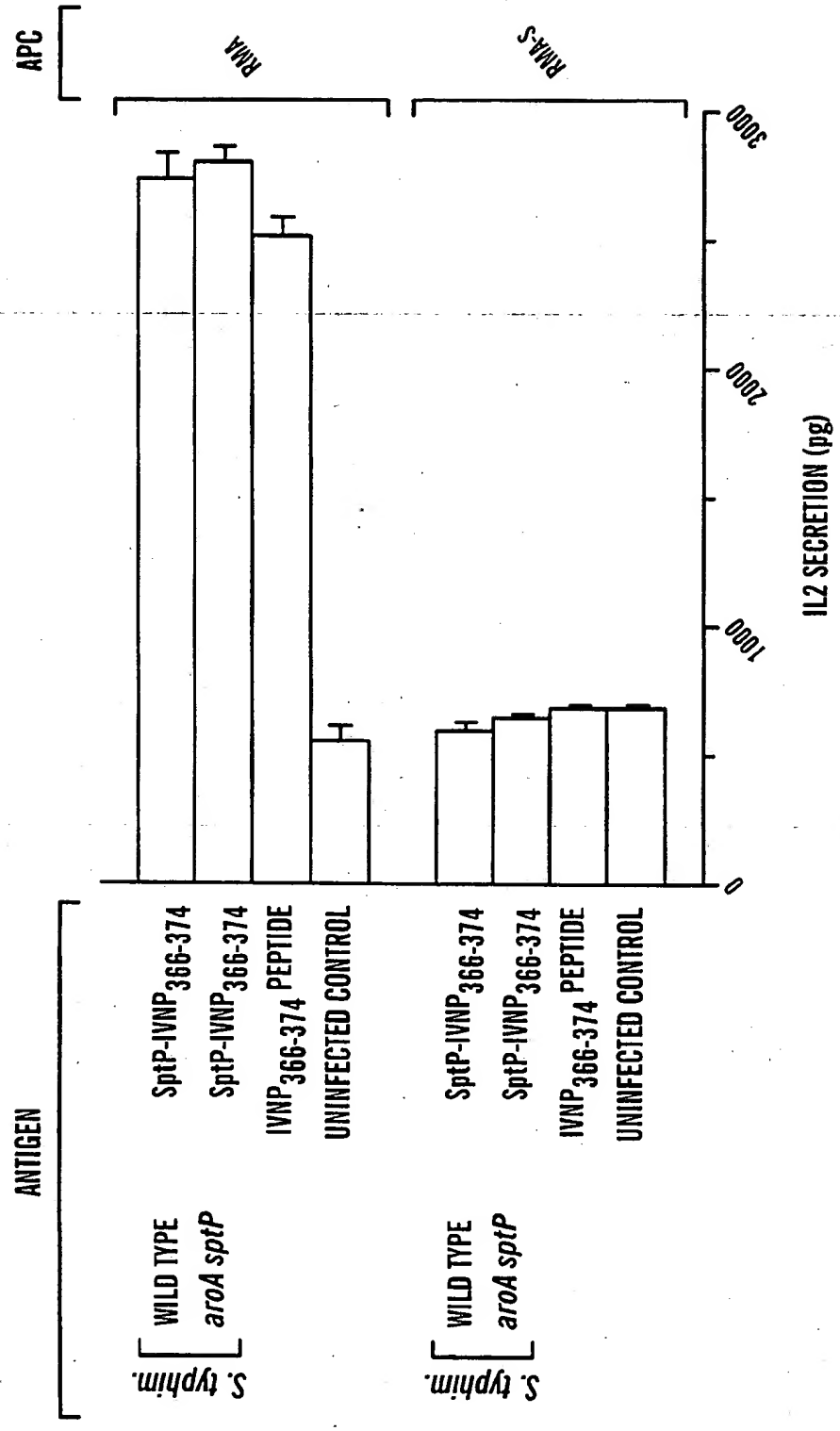
APC
RMA
RMA-S
3000
2000
1000
0
IL2 SECRETION (pg)
ANTIGEN
S. typhim.
WILD TYPE
aroA sptP
SptP-IVNP366-374
SptP-IVNP366-374
IVNP366-374 PEPTIDE
UNINFECTED CONTROL
S. typhim.
WILD TYPE
aroA sptP
SptP-IVNP366-374
SptP-IVNP366-374
IVNP366-374 PEPTIDE
UNINFECTED CONTROL

FIG. 3

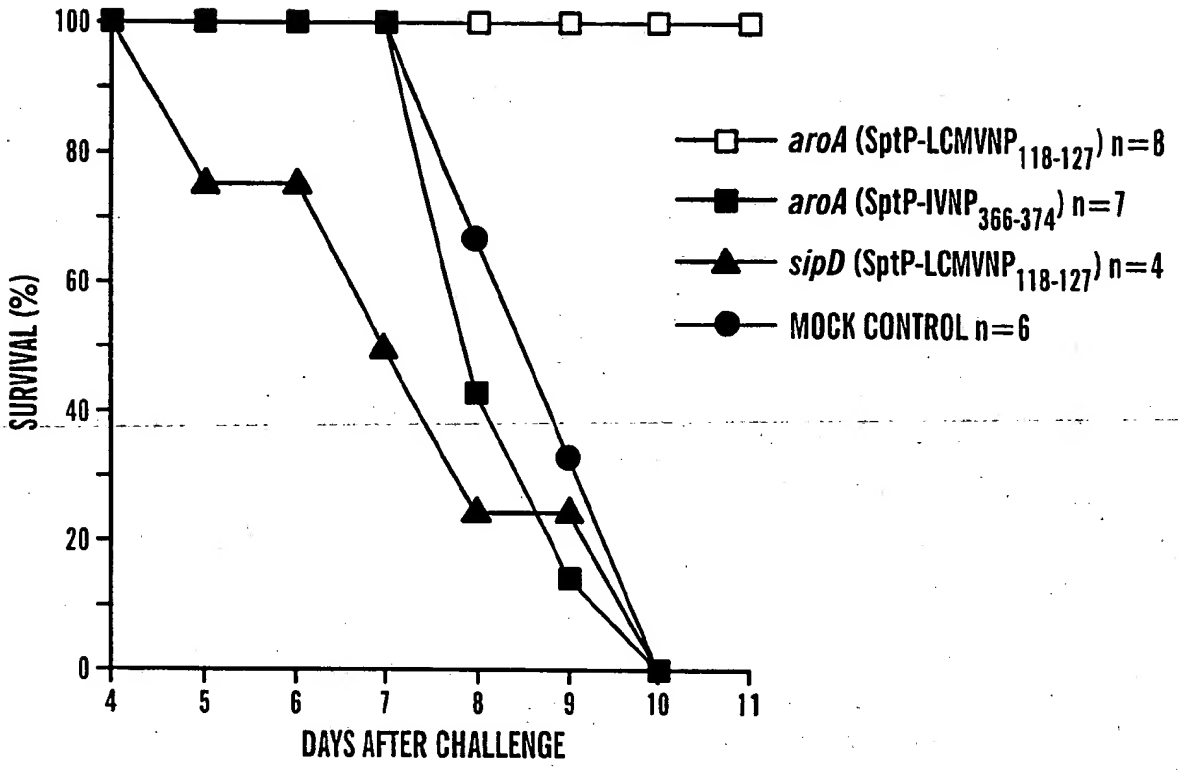
100
80
60
40
20
0
SURVIVAL (%)
4
5
6
7
8
9
10
11
DAYS AFTER CHALLENGE
aroA (SptP-LCMVNP118-127) n=8
aroA (SptP-IVNP366-374) n=7
sipD (SptP-LCMVNP118-127) n=4
MOCK CONTROL n=6

FIG. 5

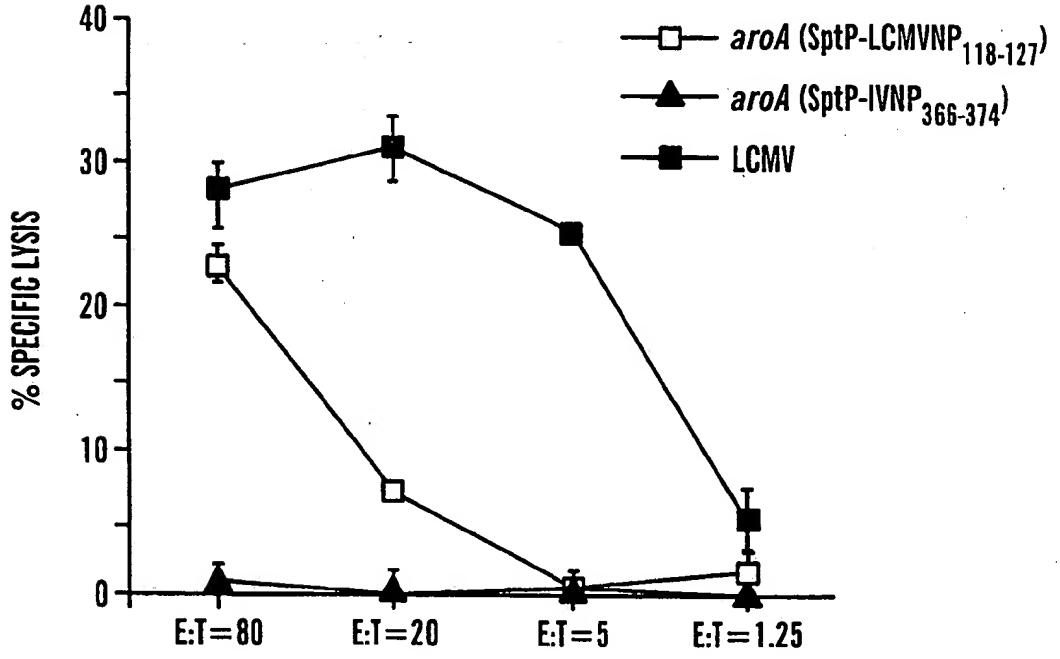
40
30
20
10
0
% SPECIFIC LYSIS
E:T=80
E:T=20
E:T=5
E:T=1.25
aroA (SptP-LCMVNP118-127)
aroA (SptP-IVNP366-374)
LCMV

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10992

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 48/00, 35/00 ; C12N 15/63, 1/21
US CL : 514/44 ; 424/93.1, 93.2 ; 435/320.1, 252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44 ; 424/93.1, 93.2 ; 435/320.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VALENTINE et al. Induction of SIV capsid-specific CTL and mucosal sIgA in mice immunized with a recombinant S. typhimurium aroA mutant. Vaccine. 1996, Vol. 14, No. 2, pages 138-146, especially pages 138-139 and 145.	1-25
Y	IKONOMIDIS et al. Delivery of a viral antigen to the Class I processing and presentation pathway by Listeria monocytogenes. J. Exp. Med. December 1994, Vol. 180, pages 2209-2218, especially pages 2209-2210 and 2215.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 JULY 1998	Date of mailing of the international search report 02 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Anne-Marie Baker, Ph.D. Telephone No. (703) 306-9155

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10992

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database AIDSLINE on STN, Accession Number 2529, ENTSCHEV et al. The Escherichia coli hemolysin secretion apparatus - a versatile antigen delivery system in attenuated Salmonella. Behring Institute Mitteilungen. 1997, Vol. 98, pages 103-113, see abstract only.	1-25
Y	COLLAZO et al. The invasion-associated type III system of Salmonella typhimurium directs the translocation of Sip proteins into the host cell. Molecular Microbiol. 1997, Vol. 24, No. 4, pages 747-756, especially pages 747, 752, and 754.	1-25
Y	HESS et al. Superior efficacy of secreted over somatic antigen display in recombinant Salmonella vaccine induced protection against listeriosis. Proc. Natl. Acad. Sci. USA February 1996, Vol. 93, pages 1458-1463, especially pages 1458, 1462, and 1463.	1-25
Y	CHENG et al. Two independent type III secretion mechanisms for YopE in Yersinia enterocolitica. Molecular Microbiol. 1997, Vol. 24, No. 4, pages 757-765, especially pages 757 and 760.	1-25
Y	KAREM et al. Protective immunity against herpes simplex virus (HSV) type 1 following oral administration of recombinant Salmonella typhimurium vaccine strains expressing HSV antigens. J. of Gen. Virol. 1997, Vol. 78, pages 427-434, especially pages 427-428 and 433.	1-25
Y	CORNELIS, G.R. The pYV plasmid, key element of Yersinia virulence. Medecine/Sciences. September 1995, Vol. 11, No. 9, pages 1295-1304, especially summary on p. 1304.	4, 5, 10, 11, 17, 18, 23, 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10992

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN - file medicine
APS
search terms: salmonella, typhimurium, vaccinat?, immuniz?, sensitiz?, stimula?, hetero?, chimer?, fusion, fuse?, ctl, class I, CD8, type III, secret?, inject?, transloc?, sptP, secretion(5n)system, immun?, T cell, Galan J/AU